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IMPACT OF FISH OIL ON INTESTINAL PERMEABILITY, INFLAMMATION, AND
PERFORMANCE IN SWINE

by

Anthony Fernando Alberto

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Animal, Dairy, and Veterinary Sciences

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2021

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ABSTRACT

Impact Of Fish Oil On Intestinal Permeability, Inflammation, And Performance In Swine

by

Anthony Fernando Alberto, Master of Science
Utah State University, 2021

Major Professor: Dr. Fernanda Batistel

Department: Animal, Dairy, and Veterinary Sciences

The objective of this study was to investigate the effect of fish oil, a source of omega-3 polyunsaturated fatty acids (**PUFA**) on intestinal permeability, systemic inflammation, and performance in piglets. An lipopolysaccharide (**LPS**) challenge was done to provoke the synthesis and release of cytokines by macrophages and neutrophils, in an effort to imitate the immune response that piglets would have when facing stress or pathogen challenge. Forty male piglets (30 days of age) were used in a complete randomized block design and assigned to one of the two treatments: 1) a control diet or 2) the control diet plus 3% of fish oil. At day 34 of the experimental period, seven animals per treatment received an LPS challenge and 24 h after intestinal tissue and digesta were collected. The statistical model included the random effect of block, and the fixed effect of treatment, day, and their interactions. There was a treatment \times day interaction for body weight and intake ($P < 0.01$). Piglets that received fish oil increased feed intake and decreased body weight over time compared with control. There was no treatment effect on body weight ($P > 0.10$); however, piglets that received fish oil had greater feed intake ($P < 0.01$). There was a treatment \times day interaction for 18-carbon fatty acids digestibility ($P =$

0.03). Piglets that received fish oil increased 18-carbon fatty acids digestibility over time when compared to the control. Compared with control, fish oil increased 16-carbons fatty acids ($P < 0.01$) and total fatty acids digestibility ($P < 0.01$). No treatment effect was observed for the cytokine TNF- α ($P = 0.63$) as well as *in vivo* intestinal permeability ($P = 0.69$) before the LPS challenge. After the LPS challenge, piglets that received fish oil tended to decrease the concentration of the inflammatory mediator PGE₂ in plasma when compared to the control ($P = 0.08$). The TNF- α concentration was increased by the fish oil after the LPS challenge ($P = 0.03$). There was a treatment \times time interaction for IL-6 on LPS challenged pigs ($P = 0.03$). After the LPS challenge, piglets that received fish oil treatment decreased the concentration of IL-6 over time when compared with control. Fish oil tended to decrease the IL-6 concentration on LPS challenged pigs, when compared with control ($P = 0.06$). No treatment effect was observed on the concentration of the inflammatory mediator LTB₄ after the LPS challenge ($P > 0.10$). Fish oil tended to decrease intestinal permeability ($P = 0.08$) in the *ex-vivo* assay after the LPS challenge. Although fish oil did not improve body weight, it had a positive effect on feed intake, systemic inflammation, and intestinal permeability after the LPS challenge. Our results suggest that supplementing an omega-3 PUFA source may modulate the immune response and promote intestinal integrity when the animals are facing an immune challenge. The protective effects of fish oil on the intestine may be closely related to preventing systemic inflammation by decreasing intestinal permeability.

PUBLIC ABSTRACT

Impact Of Fish Oil On Intestinal Permeability, Inflammation, And Performance In Swine

Anthony Fernando Alberto

Our research examined the effects of fish oil supplementation on intestinal permeability, systemic inflammation and performance in piglets. An lipopolysaccharide (LPS) challenge was done to stimulate the synthesis and release of the cytokines, in an effort to mimic the immune response that piglets would have when facing stress or pathogen challenge. Fish oil increased feed intake but did not affect growth when compared to control. Total fatty acid digestibility increased by 6% when fish oil was included in the diet. Also, we observed a 16% increase on 16-carbon fatty acids digestibility. Fish oil did not affect the plasma D-xylose concentration used to analyze *in-vivo* intestinal permeability. The plasma concentration of the pro-inflammatory cytokine TNF- α was not affected by fish oil before the LPS challenge. Immune stimulation by the LPS challenge results in the production of pro-inflammatory cytokines, which can suppress growth. After the LPS challenge, fish oil decreased PGE₂ and tended to decrease IL-6 concentration in blood samples, while TNF- α increased. A Ussing chamber was used to monitor intestinal permeability *ex-vivo*. Fish oil tended to enhance the intestinal barrier function by decreasing the FITC flux reflecting a fortified intestinal barrier function. Our results suggest that supplementing an omega-3 PUFA source may modulate the immune response and promote intestinal integrity when the animals are facing an immune challenge. The protective effects of fish oil on the intestine may be closely related to preventing systemic inflammation by decreasing intestinal permeability.

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CHAPTER 1:

REVIEW OF LITERATURE

Pork is the most globally consumed meat responsible for an estimated 23.4 billion US\$ dollars of gross output per year. As of June 2019, there were 75.5 million hogs and pigs on US farms, an increase of four percent from June 2018, according to the US Department of Agriculture's National Agricultural Statistics Service (NASS, 2019). In order to increase productivity and the number of piglets per sow per year, the weaning age has been steadily decreasing over time. The age of spontaneous weaning in domestic pigs in free-range conditions occurs between 10–12.5 weeks (Bøe, 1991), whereas, in commercial farms, piglets are weaned between 14 and 30 days (Moeser et al., 2017). However, the decrease in weaning age may present problems concerning nutrition, immunity, housing, health, behavioral and environmental requirements of the young animals (Jayaraman and Nyachoti, 2017).

Weaning is a stressful period characterized by dysfunctions on the intestinal and immune system, resulting in reduced body weight gain ascribed to low feed intake, pathogen vulnerability, and intestinal permeability (Lallès et al., 2007; Huber et al., 2018). Physiological responses to stress can significantly alter the intestinal epithelium and its barrier function, leading to inflammation and immune system activation (Kim et al., 2013; Shin et al., 2017). In the past, to mitigate the adverse effects of weaning, piglets were often fed diets containing antibiotics.

A report from the United States Department of Agriculture indicated that in 2016, 95.5% of the swine operations used antibiotics in feed, water, or injection. The use of antibiotics has a positive effect on growth rate (+16.40%) (Cromwell, 1999), diet digestibility (+5.1%) (Hardy, 1999) and feed to pound of gain ratio (+7%) (Van Lunen, 2003). Other advantages of antibiotics use can also include reducing animal weights and sizes variability, which avoids financial penalties at markets for animals outside the range suited for mechanized processing (Miller et al., 2005). However, from a public health perspective, the extensive use of antibiotics in livestock production can support microbial antibiotic resistance (Bager et al., 1997; Founou et al., 2016). As a result, the FDA set new rules to limit the utilization of antibiotics as feed additives and growth promoters in the swine industry (FDA, 2013). Thus, there is a high demand for alternatives to replace the antibiotics and alleviate the deleterious effects of early weaning in piglets.

In this review we discuss nutrient digestion, inflammation and intestinal permeability in swine. Also, we propose the use of omega-3 polyunsaturated fatty acids (PUFA) as an alternative to alleviate the effects of early weaning in piglets.

The Gastrointestinal Tract and Nutrient Digestion

The gastrointestinal tract is responsible for extracting nutrients from feed through digestion and fermentation, allowing their absorption and incorporation into the blood circulation. In swine, the gastrointestinal tract is categorized into the mouth, stomach, small intestine, and large intestine. The mouth is where nutrient digestion begins. The

mastication and secretion of salivary amylase and lipase, are the first steps for carbohydrate and fat digestion.

The stomach is anatomically located between the esophagus and the small intestine. The stomach's crucial role is to mechanically and enzymatically break down the ingested feed to be further digested by the following portions of the gastrointestinal tract (Camilleri and Vazquez Roque, 2014). The stomach releases gastric juices containing hydrochloric acid and the enzyme pepsin, which initiate the breakdown of the protein. Also, gastric lipase starts to break down triacylglycerols into diglycerides and fatty acids but very little fat digestion occurs in the stomach. No further chemical breakdown occurs for carbohydrates, because the salivary amylase does not function in the acidic conditions of the stomach. Furthermore, the mechanical breakdown is ongoing with strong stomach peristaltic contractions.

The duodenum is the first and shortest section of the small intestine. The duodenum is where secretions from the liver and pancreas are released into the small intestine and is the main site of digestion of nutrients. The combination of the pancreatic juice containing trypsin, chymotrypsin, carboxypeptidase, sodium bicarbonate and aminopeptidase results in the breakdown of most of the proteins into amino acids, di-peptides, and tri-peptides (Bröer, 2008). The polysaccharide degradation and hydrolysis process involve α -amylase secreted by the pancreas and the maltase, sucrose, and lactase from the brush border (Gray, 1992). Because the enterocytes are only capable of absorbing low-molecular-weight compounds, most of the feed's carbohydrates are broken down into monosaccharides for further absorption. The main site of fat digestion is the

duodenum with the release of the bile salts and pancreatic lipase. The lipase breaks down the triglycerides into free fatty acids and monoglycerides. Bile salts emulsify these fatty acids to facilitate their absorption through the jejunum lining.

The jejunum is the middle section of the small intestine and its lining contains long villi and microvilli increasing the contact between the digestive bolus and small intestine to optimize absorption of monosaccharides, amino acids and fatty acids (Mosenthin, 1998). The final section of the small intestine is the ileum, and its function is to absorb the nutrients that escape the jejunum's absorptive process, emphasizing bile acid reabsorption (Ticho et al., 2019). The ileum is connected to the large intestine at the ileocecal valve, controlling the chyme movement into the large intestine.

The pig large intestine is categorized by a short cecum, a long colon, and a rectum. The cecum is a blind diverticulum that extends from the colon at the ileocecal valve. The cecum's primary functions are to absorb fluids and salts that remain after completing the small intestinal digestion. The colon consists of three segments: ascending, transverse and descending colon. The colon's main function is to ferment the undigested feed that arrives from the small intestine. The colon's mucosa has no villi, but columnar epithelial cells with microvilli formed into straight tubular crypts (Bach Knudsen et al., 2012). The colon secretes large amounts of alkaline fluid to facilitate microbial fermentation. The end-products of the microbial fermentation are absorbed in the large intestine. Colonic bacteria are particularly essential to the absorption of vitamin K, vitamins B, electrolyte, and minerals (Kiela and Ghishan, 2016).

The main end-products of colonic fermentation are volatile fatty acids (**VFA**), mainly, acetate, propionate, and butyrate. The mucosa absorbs about 95% percent of the VFAs produced. These VFAs acids are used as energy for the colonocytes or taken by the mesenteric veins and transported via the portal vein to the liver to various peripheral tissues for further metabolism (Wong et al., 2006). Acetate is the most abundant VFA in the bloodstream, mainly because it is transported into various peripheral tissues for their utilization; butyrate is the preferred nutrient for the intestinal epithelial cell metabolism and development, and propionate is used as a substrate for gluconeogenesis in the liver (Bergman, 1990).

The physiological role of VFA is broader than a local effect on the intestinal enterocytes and digestive function; they indeed play a significant immunological role both systematically and locally (Tan et al., 2014). The VFA reinforce the epithelial barrier by affecting the mucus layer, epithelial cell survival, and tight junction proteins (Peng et al., 2009). Moreover, the VFA are potent anti-inflammatory mediators by inhibiting the release of pro-inflammatory cytokines from macrophages and neutrophils (Tan et al., 2014). Nevertheless, several antimicrobial activities were attributed to VFA, including disruption of osmotic and pH balance, nutrient uptake and energy generation, operating in working concentration below the toxicity threshold of host cells (Dewulf et al., 2011). Inflammation-related changes in intestinal morphology may have implications for intestinal mucosal functions, including digestion and absorption of nutrients. Thus, it is important to consider the effect of gastrointestinal inflammation on animal health to

develop nutritional strategies to maximize growth performance and pig health (Liu, 2015).

Intestinal Barrier

During the course of nutrient absorption, the intestine is challenged by the food antigens, invading microorganisms, and also the resident bacteria (Ulluwishewa et al., 2011). Thus, besides being permeable for the nutrients, it provides an effective barrier against antigens and pathogens (Janeway, 1999). Approximately a quarter of the intestinal mucosa is constituted by lymphoid tissue, and about 70% of the immune cells are located within the intestine (Mackie et al., 1997). The intestinal barrier has three types of defense, the biological barrier, which is made up of the gastrointestinal microbiota responsible for the colonization of the gastrointestinal tract; the immune barrier, that comprises the lymphoid tissue related to the intestine, such as macrophages, antibodies, and lymphocytes contained by the lamina propria and the physical barrier that is regulated by the tight junctions (Assimakopoulos et al., 2011).

The intestine mediates nutrient absorption by two major routes: transcellular and paracellular pathways. Transcellular permeability is generally associated with solutes transported through the cells and is regulated by selective transporters for amino acids, monosaccharides, and fatty acids (Groschwitz and Hogan, 2009). Paracellular permeability is associated with the transport of hydrophilic substances across the epithelium by passing through the space between the enterocytes; this transport process is regulated by protein complexes located in the apical and lateral membrane junction (Groschwitz and Hogan, 2009). The paracellular pathway is thought to play a minor role

in absorption of glucose, secondary metabolites in foods (Karasov, 2011) drugs, and medium molecular weight amino acids (Bröer, 2008).

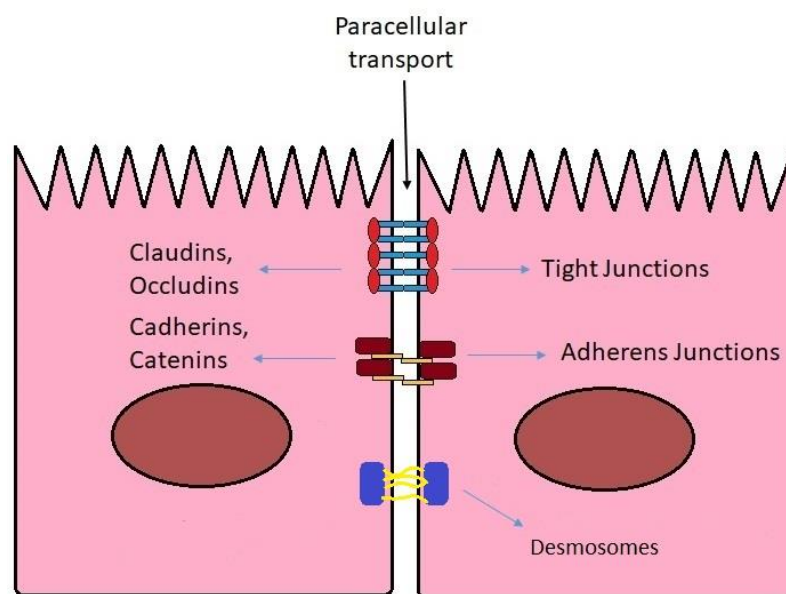
The intestine's epithelium cells are kept together by three protein complexes: desmosomes, adherens junction, and tight junctions (**Figure 1-1**) (Turner, 2009).

Desmosomes provide mechanical strength to the epithelium that is important for the peristalsis of the gut (Luissint et al., 2016). The tight junctions form a belt-like adhesive seal between the enterocytes. Immediately below the tight junctions are adherens junctions that mediate strong cell-to-cell adhesion. Little is known about the role of desmosomes in the barrier function. However, the tight junctions and adherens junctions play a crucial role in the adhesion of the epithelium and subsequent barrier function of the tissues, especially the small intestine (Niessen, 2007).

The tight junctions selectively limit the transport of solutes through the intestine, especially the macromolecules that are not absorbed via the transcellular pathway (Lee et al., 2018). The main transmembrane proteins forming strands to keep the enterocytes together are claudins, which are essential for tight junction formation and function (Assimakopoulos et al., 2011). Tight junctions also contain a second major transmembrane protein called occludins; these are important to keep the enterocytes together and limit the junction permeability (Al-Sadi et al., 2011). Claudins and occludins are associated with intracellular peripheral membrane proteins called zona occludens proteins (Blackstone, 2003). The tight junctions have an essential effect on health; its disruption increases intestinal permeability and has been associated with diarrhea, systemic inflammation, and other pathogenic effects (Guttman and Finlay, 2009). The

intestine of young piglets is immature, and infections, various stressors and feed-related factors can alter the function of the intestinal barrier (Wang and Ji, 2019). Omega-3 PUFA are nutrients that can strengthen the integrity of the barrier and counteract infections through the expression and distribution of tight junctions (Krizak et al., 2016).

Figure 1-1. Illustration of tight junctions, adherens junctions and desmosomes in the enterocytes



The Gastrointestinal Microbiota

Immune Response and Animal Performance

The interaction between host and external environment mainly occurs in the gastrointestinal tract. The mucosal barrier has a critical role in many physiologic functions ranging from digestion, absorption, and metabolism but also tightly regulates the passage of pro-inflammatory molecules, microorganisms, toxins, and antigens (Farré et al., 2020). Inflammation of the epithelium leads to an impaired barrier function that

could cause deleterious consequences such as sepsis and endotoxemia (Faintuch and Faintuch, 2019). The severity and period of the inflammatory response, there may shift in the flux of nutrients away from growth and more toward immune support (Klasing and Johnstone, 1991). While the reduction in animal performance during an immune response are primarily associated with decreased feed intake, the increased nutrient need for the immune system may help explain why digestive bacterial infections result in the most significant reduction in growth relative to other immune challenges (Pastorelli et al., 2012).

Prostaglandins and cytokines mediate symptoms of sickness and inflammation. Prostaglandins play a crucial role in the generation of the inflammatory response; their biosynthesis is significantly increased in inflamed tissue, contributing to inflammation development (Ricciotti and FitzGerald, 2011). Cytokines are proteins made by cells that affect the behavior of other cells. The cytokines secreted by macrophages play essential roles in innate responses (Arango Duque and Descoteaux, 2014). These cytokines include $\text{TNF-}\alpha$, $\text{IL-1}\beta$, and IL-6 (Janeway, 1999). These pro-inflammatory cytokines also have other biological activities that help coordinate the body's responses to infection.

The immune system activation results in the release of pro-inflammatory mediators and signaling molecules to help neutralize the infection locally. At the site of inflammation, IL-1 , IL-6 , and $\text{TNF-}\alpha$ produced by macrophages/monocytes can activate endothelial cells, upregulate their expression of adhesion molecules, and enhance attachment/migration leukocytes into the inflamed tissue (Liu and Ahearn, 2005). When a localized inflammation can not be controlled, cytokines released at the site of infection

proceed to recruit other immune cells into the region, and the inflammatory mediators are released into the bloodstream, which is clinically identified as a systemic inflammation (Lallès et al., 2007). The presence of systemic inflammation during weaning may play a role in growth suppression, anorexia, and overall pig health. Pro-inflammatory cytokines are deleterious for the intestinal integrity and epithelial function, that are related to permeability and transport of nutrients (McKay and Baird, 1999).

Omega-3 Polyunsaturated Fatty Acids

Omega-3 fatty acids are a group of PUFA that includes α -linolenic acid, eicosapentaenoic acid (**EPA**), and docosahexaenoic acid. The omega-3 PUFA receive their name based on a structural description, referring to the double bond position that is closest to the methyl terminal. Omega-3 PUFA are gaining importance in animal production systems to improve animals' health and productivity. Omega-3 PUFA are proving indispensable in numerous of biological, physiological, developmental, reproductive, and beneficial health functions (Konieczka et al., 2017, Lee et al., 2019).

The anti-inflammatory properties of omega-3 PUFA are supported by their ability to inhibit production of inflammatory mediators, including eicosanoids such as prostaglandin E₂ (**PGE₂**) and leukotriene B₄ (**LTB₄**), and pro-inflammatory cytokines tumor necrosis factor- α (**TNF- α**), interleukin-1 β (**IL-1 β**), and interleukin-6 (**IL-6**) (Kromhout et al., 2012; Darwesh et al., 2019). Eicosanoids are a group of chemical messengers that act within the immune system involved in modulationg the intestity and duration of inflammatory responses. Eicosanoids include prostaglandins, thromboxanes,

leukotrienes, and lipoxins. Prostaglandins have several proinflammatory effects, including induction of fever and erythema, increasing vascular permeability and vasodilation, and enhancing pain and edema caused by other agents such as histamine (Calder, 2001).

Omega-3 PUFA appear to inhibit arachidonic acid (AA) derived pro-inflammatory mediators such as PGE₂ and LTB₄ by changing the pathway towards PGE₃ and LTB₅ which are less inflammatory (James et al., 2000). Since EPA acts as a substrate for the enzymes that synthesize prostaglandins, an increased intake of omega-3 PUFA would lead to an elevation in the production of EPA-derived eicosanoids mirrored by a suppression of proinflammatory AA-derived eicosanoids (Calder, 2001). This is supported by an experiment done by Walker et al. (2015) where feeding humans increased amounts of PUFA resulted in decreased AA in the cells' membrane.

The amount of PUFA in immune cells provides is a possible link between PUFA intake, immunity, and inflammation. Supplementation of omega-3 PUFA on piglets had a positive effect on growth, possibly by decreasing inflammatory cytokines (**Table 1-1**). In addition to inhibiting pro-inflammatory mediators, omega-3 PUFA has also proven to improve intestinal barrier function. Whiting et al. (2005) fed an omega-3 PUFA rich diet to mice, and saw that it promoted intestinal barrier maintenance. In this same order, the supplementation of omega-3 PUFA on weaned animals may improve the health status of the weaning period in which animal's different stressors due to their effects on the immune system (Upadhaya et al., 2019).

Table 1-1. Summary of the effects of omega-3 PUFA on growth, nutrient digestibility, and inflammation in swine.

Item	Growth	Digestibility	Inflammation mediators
Upadhaya et al., 2019	+6 g/d	2%	=
Liu et al., 2003	=	-	↓ PGE ₂
Shin et al., 2017	+25g/d	-	↓ TNF- α
Upadhaya et al., 2017	+25 g/d	=	-
Duan et al., 2014	+4 g/d	-	=
Gaines et al., 2003	=	-	↓ TNF- α
Carroll et al., 2003	=	-	↓ TNF- α

(-) means not measured by the authors, (=) no statistical difference, (↓) decreased.

Conclusions

Dietary fatty acids provide energy for growth and production of pigs.

Furthermore, it has been shown that omega-3 PUFA can modulate the immune and inflammation responses, and improve the integrity of the intestinal barrier.

Understanding better this phenomenon can help a swine nutritionist include these in the diet to meet the energy requirement and the benefits of its fatty acid content. Omega-3 PUFA have been shown to have anti-inflammatory properties; however, to our knowledge, intestinal permeability has only been evaluated in newborn piglets.

Therefore, determining the impact of fish oil on piglets is of particular importance because it can provide an important asset to ameliorate the effects of weaning on the pigs' overall health.

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CHAPTER 2: IMPACT OF FISH OIL ON INTESTINAL PERMEABILITY, INFLAMMATION, AND PERFORMANCE IN SWINE

Introduction

Stress occurring during the weaning period can have long-lasting effects on gastrointestinal barrier health (Smith et al., 2010). Weaning incorporates many stressors (e.g., maternal separation, transportation, the stress of combining, shift of diet, etc.) that lead to deleterious impacts on performance, gastrointestinal function and increased susceptibility to infectious diseases (Moeser et al., 2017). The number of significant diseases associated with intestinal permeability, including chronic inflammatory, and functional disorders and life-threatening conditions such as sepsis and multiple organ dysfunction, highlights the critical importance of proper regulation of epithelial permeability (Camilleri and Gorman, 2007). A study done by Spreeuwenberg et al. (2001) observed that 4 days after weaning, pigs with increased intestinal permeability and villous height also had a lower feed intake.

Fatty acids are gaining importance in animal production because of their potential effects on animal health and productivity (Cherian, 2015; Simopoulos, 2016). Omega-3 polyunsaturated fatty acids (**PUFA**) such as docosahexaenoic acid (**DHA**) and eicosapentaenoic acid (**EPA**), are indispensable in numerous physiological, developmental, reproductive, and immune functions (Konieczka et al., 2017; Lee et al., 2019). The proportion of PUFA in immune cells provides a link between PUFA intake, immunity, and inflammation (Calder, 2001). The anti-inflammatory properties of omega-3 PUFA are supported by their ability to inhibit production of inflammatory mediators, including eicosanoids prostaglandin E₂ (**PGE₂**) and leukotriene B₄ (**LTB₄**), and pro-

inflammatory cytokines tumor necrosis factor- α (**TNF- α**), interleukin-1 β (**IL-1 β**), and interleukin-6 (**IL-6**) (Kromhout et al., 2012; Darwesh et al., 2019). Increased cytokine activity results in decreased feed intake and growth in immunologically challenged pigs (Kelley et al., 1994). The inclusion of 7% fish oil to LPS-challenged piglets decreased inflammatory cytokines, including TNF- α (Liu et al., 2003). Inclusion of 7% fish oil in the feed of sow's one week prior to conception resulted in a decreased production of proinflammatory cytokines in the offspring (Fritsche et al., 1993). Furthermore, Lam et al. (2015) observed enhanced intestinal barrier function in mice with 37% of the fat being omega-3 PUFA. This suggests that consumption of a diet rich in omega-3 PUFA may confer potential health benefits by modulating inflammatory response and promoting the intestinal health in pigs (Upadhaya et al., 2019).

The objectives of this study were to investigate the effect of fish oil, a source of omega-3 PUFA, on intestinal permeability, systemic inflammation, and performance in piglets. An LPS challenge was done to provoke the synthesis and release of cytokines by macrophages and neutrophils, in an effort to imitate the immune response that piglets would have when facing stress or pathogen challenge. We hypothesized that supplementing fish oil would enhance intake and animal growth by decreasing intestinal permeability and consequently systemic inflammation. Furthermore, we hypothesized that the addition of fish oil could enhance the intestinal barrier function and decrease circulatory pro-inflammatory cytokines in the blood after the LPS challenge.

Materials and Methods

The animals used in this study were cared for according to the Institutional Animal Care and Use Committee at Utah State University. The study was conducted at the Utah State University Animal Science Farm (Logan, UT) from March 28, 2020 to May 4, 2020.

Experimental Design and Dietary Treatments

Forty male Landrace x Hampshire piglets (body weight 8.2 ± 0.83 kg, age 28 days) were bought from a commercial farm. They were randomly assigned to one of two treatments in a randomized complete block design: a basal pig starter diet without fish oil (**Control**; IFA Country Store, Logan, UT) and the control diet plus 3% of fish oil (dry matter basis) (**Fish Oil**; Omega Protein Inc., Reedville, VA). The experimental diets were ground and delivered daily at 8:00 am for 5 weeks. The basal diet met the animal's requirements for all the nutrients (NRC, 2012). Pigs were housed in 3 pens (2 pens containing 7 piglets and 1 pen with 6 piglets) per treatment and each pen was equipped with a feeder and a nipple waterer.

Data collection, sample and analysis

Body weight was recorded weekly throughout the 5-week experiment. Feed samples were pulled weekly and composited for the duration of each period and for dry matter analysis.

D-xylose, to measure *in-vivo* intestinal permeability, was orally administered at 8:30 am on days 7, 14, 21 and 28 of the experimental period at a dose of 0.1g/kg (Hou et

al., 2012). Blood samples (~10 mL) were collected 3 hours after the D-xylose administration. Samples were taken via anterior vena cava puncture into evacuated tubes (BD Vacutainer, BD and 52 Co., Franklin Lakes, NJ) containing lithium heparin and centrifuged (3,000 x g for 15 min) to separate plasma. The plasma samples were stored at -80°C until analysed. D-xylose in plasma was quantified using a commercially available ELISA kit (Catalog no. MBS755704, MyBioSource, San Diego, CA) according to the manufacturer's instructions. Also, blood samples were taken with tubes containing heparin on days 13, 20, 27 to analyze TNF- α concentration using a commercially available ELISA kit (Catalog no. KSC3012/KSC3011, Invitrogen Corporation, Carlsbad, CA).

Titanium dioxide was added to the diets at 0.30% concentration to record individual feed intake. Fecal samples were collected via rectal massage for 5 consecutive days on the 2nd and 4th week. Samples were thawed and composed by piglet, dried in a 65°C forced air oven for 48-72 h, and ground through a 1-mm screen with a Wiley mill. Titanium dioxide (**TiO₂**) concentrations were used to estimate fecal output. The procedure to determine the TiO₂ in the feces was performed according to Myers et al. (2004). Total fecal excretion and feed intake were calculated as follows: 1) Total fecal excretion = amount of TiO₂ dosed (g/d)/concentration of TiO₂ in feces (g/kg); and 2) Intake = total fecal excretion (kg/d)/feed indigestibility. Fatty acid digestibility was measured in the feces with a 2-step methylation procedure, as described by Jenkins (2010).

At the end of the experiment, 7 piglets per treatment received intraperitoneal administration of LPS (*Escherichia coli* serotype 055:B5; Catalog no. L2880, Sigma Chemical Inc., St. Louis, MO) at the dose of 80 µg/kg BW (Hou et al., 2010). The LPS was done to provoke the synthesis and release of cytokines by macrophages and neutrophils, in an effort to imitate the immune response that piglets would have when facing stress or pathogen challenge. Blood samples were collected on tubes containing heparin, 3h and 24h after the LPS challenge, to analyze immune mediators TNF- α , IL-6, PGE₂, and LTB₄. The concentrations of IL-6 (Catalog no. ESIL6, Life Technologies, Carlsbad, CA), PGE₂ (Catalog no. MBS265747, MyBioSource), LBT₄ (Catalog no. MBS284817, MyBioSource), and TNF- α (Catalog no. KSC3012/KSC3011, Invitrogen Corporation) were analyzed according to the manufacturer's instructions.

Twenty-four hours after the LPS, the piglets were euthanized under anesthesia with an intravenous injection of sodium pentobarbital (50 mg/kg BW). The intestine was dissected and 5-cm intestinal segments of the jejunum and ileum were placed on ice until analysis. The *ex-vivo* intestinal permeability measured paracellular flux of fluorescein isothiocyanate dextran 4 kDa (**FITC**) in Ussing chambers. The segments of jejunum and ileum were oxygenated in a (95% O₂/5% CO₂) Ringer's solution, and then mounted in Ussing chambers (model U-2500, Warner Instruments, Hamden, CT). After a 15-min equilibration, the probe FITC (catalog no. 60842-46-8, Sigma-Aldrich, St. Louis, MO) was added to the mucosal side at the final concentration of 0.375 mg/mL (Hu et al., 2013). Mucosal-to-serosal flux of FITC was performed by sampling 100 µL of solution from the serosal side, 30-min after the the probe was added. The concentrations of FITC

in the serosal side were measured by fluorescence microplate reader as previously described by Watson et al. (2015). Furthermore, digesta samples from the jejunum, ileum, cecum and, colon were collected, 1 mL of 6 N HCl was added to stop fermentation, and then frozen at -80°C for VFA analysis. Samples were analyzed according to Eun and Beauchemin (2007).

Statistical Analysis

Data were analyzed using the MIXED procedure of SAS v.9.4 (SAS Institute, Inc. Cary, NC) according to the following model:

$$Y_{ijk} = \mu + b_i + T_j + D_k + TD_{jk} + e_{ijk}$$

Where Y_{ijk} is the variable of interest, μ is the pen overall mean, b_i is the random effect of block, T_j is the fixed effect of treatment, D_k is the fixed effect of day, TD_{jk} is the interaction between treatment and day, e_{ijk} is the residual error. Data for volatile fatty acids and Ussing chamber was analyzed replacing the variable time by tissue in the model. The normality of the residuals was checked with normal probability and box plots and homogeneity of variances with plots of residuals vs. predicted values. Significance was determined at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

Results

Animal production

There was a treatment \times day interaction for feed intake and body weight ($P < 0.01$; **Figure 2-1** and **Figure 2-2**). Piglets that received fish oil increased feed intake and decreased body weight over time compared with control. No treatment effect was observed for final body weight (11.9 vs 11.3 kg; $P > 0.10$; **Table 2-3**) or average daily gain (**ADG**) (131 vs 115 g; $P > 0.10$). Piglets that received fish oil had greater overall feed intake per day (1049 vs. 729 g; $P < 0.01$) when compared with control.

Fatty Acids Digestibility

There was a treatment \times day interaction for 18-carbon fatty acids digestibility ($P = 0.03$; **Figure 2-3**). Piglets that received fish oil increased 18-carbon fatty acid digestibility over time when compared to the control. The concentration of 18-carbon fatty acids was not affected by treatments or time ($P > 0.10$). There was not treatment \times day interaction for 16-carbon fatty acids or total fatty acids digestibility ($P > 0.10$). Compared with control, fish oil increased 16-carbons fatty acids (73.4% vs 57.4%; $P < 0.01$ and total fatty acids digestibility (78.4% vs. 71.2 %; $P < 0.01$). There was not a time effect for 16-carbons fatty acid digestibility ($P > 0.10$); however, total fatty acids digestibility increased over time for both treatments ($P < 0.01$).

Circulating pro-inflammatory cytokines and in-vivo permeability

There was no treatment \times day interaction for TNF- α ($P > 0.10$; **Figure 2-4**) and D-xylose ($P > 0.10$; **Figure 2-5**). No treatment effect was observed for the cytokine TNF- α ($P > 0.10$) as well as *in vivo* intestinal permeability ($P > 0.10$). No time effect was observed for D-xylose absorption or TNF- α concentration ($P > 0.10$).

Volatile fatty acids concentration after the LPS challenge

There was no interaction between treatment \times tissue for volatile fatty acids concentration after the LPS challenge ($P > 0.10$). The concentration of VFAs in the gastrointestinal tract after the LPS challenge was not affected by treatments ($P > 0.10$). The acetate concentration was higher in the small intestine, when compared with control, while the concentration of butyrate and propionate was higher in the large intestine. There was a higher concentration of acetate in the jejunum and ileum when compared to the cecum and colon ($P < 0.01$; **Figure 2-6**). The concentrations of propionate and butyrate were higher in the cecum and colon, when compared to the jejunum and ileum ($P < 0.01$).

Circulating pro-inflammatory cytokines and ex-vivo intestinal permeability after the LPS challenge

There was no treatment \times time interaction for PGE₂ ($P > 0.10$; **Table 2-4**) after the LPS challenge. The piglets that received fish oil had a tendency to decrease the PGE₂ concentration in plasma, when compared to the control (72.8 vs 64.7 pg/ml, $P = 0.08$; **Figure 2-7**). Both treatments had a tendency to decrease PGE₂ on plasma over time ($P = 0.08$). No treatment \times time interaction was observed for LTB₄ concentration ($P > 0.10$). The treatments did not have an effect on LTB₄ concentration ($P > 0.10$); however, the LTB₄ concentration decreased over time for both treatments ($P = 0.05$). There was no treatment \times time interaction for the cytokine TNF- α ($P > 0.10$). The fish oil supplementation increased the concentration of TNF- α when compared to control (817 vs 1318 pg/mL, $P = 0.03$; **Figure 2-7**). The concentration of TNF- α in plasma samples decreased over time for both treatments ($P < 0.01$). There was a treatment \times time interaction for IL-6 ($P = 0.03$). Piglets that received fish oil decreased IL-6 concentration

over time when compared with control (**Figure 2-7**). The fish oil supplementation tended to decrease the IL-6 concentration in plasma, when compared with control (565 vs 395 pg/ml, $P = 0.06$). Both treatments decreased the IL-6 concentration over time ($P = 0.03$).

The FITC concentration for *ex-vivo* intestinal permeability had no treatment \times tissue interaction, after the LPS challenge. However, the type of tissue analyzed (jejunum and ileum) had an effect over the FITC concentration ($P = 0.05$; **Figure 2-8**). Fish oil tended to decrease intestinal permeability of the jejunum after the LPS challenge, when compared to control (6.63 vs 6.97 pg/ml, $P = 0.08$).

Discussion

Weaning of pigs involves complex events, including environmental and dietary stresses that interfere with gastrointestinal development and adaptation (Lallès et al., 2007). The small intestine's biochemical changes cause excessive secretion of pro-inflammatory cytokines and induce severe intestinal inflammation (Li et al., 2014). Fish oil has a high content of PUFA, especially omega-3 PUFA, which have shown anti-inflammatory benefits in previous studies in humans (Calder and Grimble, 2002) and swine (Mateo et al., 2009; Liu et al., 2003). To test our hypothesis that omega-3 PUFA can enhance growth, decrease inflammation and improve intestinal barrier function. We examined the effects of fish oil on growth, systemic inflammation and intestinal permeability on piglets. Furthermore, an LPS challenge was done to provoke macrophages and neutrophils to synthesize and release cytokines in an effort to imitate the immune response that piglets would have when facing stress or pathogenic challenge.

In our experiment, the inclusion of 3% of fish oil increased the piglets' feed intake, but there was no effect in body weight gain. Li et al. (2014) obtained similar results; they fed 3% of a source that contained 33% of omega-3 PUFA to weaned piglets for 28 days and observed no effect on body weight gain or feed efficiency. Eastwood (2008) fed different levels of flaxseed meal (0, 100, 200, and 300 g of flaxseed meal/kg of diet), which had 65g/kg of α -linolenic acid, and observed no linear effects on basal body weight gain. In contrast, Upadhaya et al. (2019) observed that the supplementation of varied omega-3 to omega-6 PUFA ratios (15:1, 10:1, and 5:1) in the diet led to a linear increase in body weight gain and average daily gain on days 28 to 49. In a study in rats, Baillie et al. (1999) observed that less dietary energy is stored as fat when rats are fed a diet containing fish oil compared with corn oil. In a study done by Kavanagh et al. (2001), TiO₂ recovery in feces of weaned piglets was about 90%, which shows that individual intake based on TiO₂ recovery could lead to inaccurate intake measurements. In our study, a scarce TiO₂ recovery leading to false intake measurement could possibly explain the increase in intake but not in body weight observed.

Total fatty acid digestibility increased with the supplementation of 3% of fish oil. Also, the C16 fatty acid digestibility increased with addition of fish oil. The improved digestibility of fatty acids based on an omega-3 PUFA source in weaned piglets has not been reported; thus, comparison within the same species could not be made. However, Skřivan et al. (2018) obtained similar results in chickens where he fed 60g/kg of different types of fat and showed an increase in fat digestibility. The increase in fatty acid digestibility may be due to the increase in fat content in the treatment diet. Tancharoenrat

et al. (2014) reported that unsaturated fatty acids were well digested irrespective of the source of fat in chickens. A similar study by Hurwitz et al. (1979) in turkeys reported that the digestibility estimates for unsaturated fatty acids (oleic and linoleic acids) were higher than those for saturated fatty acids (palmitic and stearic acids). This could explain the difference in fatty acids digestion in the current experiment, as the fish oil has a higher concentration of unsaturated fatty acids. Once digested, the unsaturated free fatty acids form micelles along with the conjugated bile acids. In contrast, the saturated fatty acid incorporation into the micelles is limited because of their non-polarity, which makes them depend on an adequate presence of bile salts and unsaturated fatty acids for efficient emulsification and further digestion (Polin et al., 1980).

Previous studies suggest the inclusion of omega-3 PUFA in the diet could significantly improve the piglet's immune status and decrease inflammation. Tumor necrosis factor- α is thought to be one of the major inflammatory cytokines. In the present study, plasma TNF- α concentrations were not affected by the fish oil. These results are consistent with Shin et al. (2017), where they fed different omega-6 to omega-3 ratios to weaning piglets in a challenging environment (no cleaning or disinfection of a previously populated room) and there was no difference between the treatments. Nonetheless, some *in vivo* studies in pigs, observed that omega-3 PUFA decreased TNF- α production after an LPS challenge (Liu et al., 2003; Mateo et al., 2009). Since the samples of our TNF- α assay were taken before the LPS challenge, this result was expected because pigs in a healthy environment would not trigger inflammatory responses.

The intestinal barrier integrity during the weaning period is important for optimal digestion and absorption of nutrients. Weaning stressors and microbial pathogenic exposure typically increase villous atrophy and hyperplasia of the crypts. The intestinal barrier plays a critical role in preventing luminal bacteria's entrance and allergens from the diet into the mucosa. D-xylose absorption proves to be a useful marker to measure *in vivo* intestinal integrity in piglets (Pluske et al., 1996; Berkeveld et al., 2008). In the present experiment, the D-xylose concentration to assess *in vivo* permeability was not different between treatments. Liu et al. (2012) reported enhanced intestinal barrier function with the inclusion of 5% fish oil to weaned piglets, indicated by decreased plasma diamine oxidase activity, compared with corn oil. Also, transmembrane tight junction protein occludin expression demonstrated only a trend for an improvement as a result of reducing the n-6:n-3 PUFA ratio in diets (Shin et al., 2017). These data suggest that under healthy conditions the omega-3 PUFA does not affect intestinal barrier function.

The concentration of VFAs was measured after the LPS challenge. The VFAs such as acetate, propionate and butyrate are the main products of microbial breakdown of carbohydrates in the large intestine (Cummings and Macfarlane, 1997). Butyrate has anti-inflammatory properties and has also shown protection against mucosal oxidative stress, and enhancement of the colonic defense barrier (Canani et al., 2011). In the present study, VFAs were not affected by the treatment, but a difference among the tissues was observed. Interestingly, the acetate concentration was higher in the small intestine. Also, Hoogeveen et al. (2020) observed a higher acetate fermentation in the small intestine

when pigs were fed a human-type diet for 14 weeks. The large intestine had a higher concentration of butyrate and propionate in the cecum and colon. A higher concentration of butyrate and propionate in the large intestine was expected, as a higher fermentation takes place in the pig's large intestine (Huang et al., 2004).

The blood circulation of the inflammatory mediators PGE₂, TNF- α , IL-6, LTB₄ and has been correlated with a pro-inflammatory response. In our experiment, PGE₂ concentration was decreased by the fish oil after the LPS challenge. In agreement with our findings, Liu et al., (2003) supplemented 7% fish oil for 14 days to LPS challenged piglets and observed decreased blood circulation of PGE₂. Similarly, Upadhaya et al. (2015) decreased PGE₂ circulation of LPS challenged finishing pigs by supplementing the diet with 0.75% omega-3 PUFA in a 6-week experiment. This results are indicate that omega-3 PUFA can shift the prostaglandin pathway towards PGE₃, which results in decreased PGE₂ synthesis, as they compete for the same cyclooxygenase (COX) enzymes.

The TNF- α circulation in blood was increased by the fish oil supplementation. Similarly, several mice studies had shown that omega-3 PUFA increase TNF- α secretion by macrophage cells (Chang et al., 1992, Carrick et al., 1994, Skuladottir et al., 2007). Moreover, our results are consistent with a finishing pigs experiment by Komprda et al. (2018) who fed a diet containing 2.5% fish oil for 70 days and observed an increased TNF- α circulation after a LPS challenge. Previous studies have demonstrated that PGE₂ are effective stimuli for the rapid induction of intracellular cAMP in a dose-dependent manner, and cAMP has shown to suppress TNF- α expression (Samuelsson et al., 1978;

Kunkel et al., 1988; Di Battista et al., 1999). The decreasing PGE₂ production is thought to increase the macrophages TNF- α secretion (Kunkel et al., 1988, Hardardottir and Kinsella, 1991). Based on this, we can speculate that fish oil by increasing PGE₃, the PGE₂ synthesis was decreased, consequently suppressing the regulatory mechanism on which TNF- α is inhibited by PGE₂. More studies are necessary to elucidate the mechanism by which PGE₂ stimulates of TNF- α .

Immune response is typically induced by the production of inflammatory cytokines. In our experiment, fish oil tended to decrease pro-inflammatory cytokine IL-6 concentration in blood samples. Duan et al. (2014) observed decreased concentration of IL-6, when different ratios of omega-3 PUFA were included in the diet (1:1, 2.5:1, 5:1, 10:1) of finishing pigs. In a colitis model, rats fed a diet rich in omega-3 PUFA had decreased colonic concentrations of IL-6 (Charpentier et al., 2018). However, fish oil did not have an effect over LTB₄, 3 h and 24 h after the LPS challenge. There is not much In a mouse model of immunodeficiency-induced colitis, 8 week fish oil supplementation had no effect on mucosal LTB₄ (Bosco et al., 2013). However, some other studies had shown a decreased LTB₄ in some other studies (Shoda et al., 1995; Hudert et al., 2006). Based on this results, the fish oil can exert beneficial effects IL-6 but more research needs to be done to elucidate the mechanisms by which omega-3 affect LTB₄.

After the LPS challenge, the Ussing chamber technique was used to monitor intestinal permeability in terms of flux of FITC marker probe. The FITC was added to the solution in the Ussing chamber on the mucosal side. The appearance of this marker probe in the chamber at the serosal side represents its permeability across the epithelium

(Overman et al., 2012). The flux of intact FITC across the intestinal epithelium occurs mainly through paracellular pathways (Hamard et al., 2010). The results of this study showed that 3% fish oil supplementation tended to enhance the intestinal barrier function by decreasing the FITC flux reflecting a fortified intestinal barrier function. Our observation was supported by Jacobi et al., (2012), who showed that supplementation of milk with 5% EPA of the total fatty acids on nursery piglets, enhanced transepithelial electrical resistance in the Ussing chamber. Also, Willemsen et al. (2008) reported that a fat blend containing 0.06% EPA and 0.30% DHA supported epithelial barrier integrity on cultured human epithelial cells by improving transepithelial electrical resistance. Mani et al. (2013) reported that omega-3 PUFA reduced *ex vivo* mucosal to serosal endotoxin transport permeability in growing pigs compared with control (no oil addition). In this experiment, we observed a trend toward improved intestinal integrity by feeding fish oil. Previous research has shown that omega-3 PUFA can modulate intestinal barrier in several ways. 1) Incorporation into the cellphospholipid membrane, 2) Modulation of tight junctions expression and redistribution, 3) Inhibition of proinflammatory mediators by producing eicosanoids with anti-inflammatory properties (Durkin et al., 2021). As PGE₂ and IL-6 inflammation mediators were decreased by fish oil supplementation. We could speculate that intestinal barrier function was enhanced by the anti-inflammatory properties of omega-3 PUFA. Our experiment suggested that under a pathogen challenge, the fish oil could improve the intestinal epithelium condition.

Conclusions

This study shows that 3% fish oil supplementation increased feed intake but did not affect animal performance, cytokine production or intestinal permeability before the animals were challenged with the LPS. However, after the LPS challenge, fish oil exerted beneficial effects over systemic inflammation and tended to decrease intestinal permeability. Our results suggest that supplementing an omega-3 PUFA source may modulate the immune response and promote intestinal integrity when the animals are facing a immune challenge. The protective effects of fish oil on the intestine may be closely related to preventing systemic inflammation by decreasing intestinal permeability.

Tables**Table 2- 1. Nutrient levels of the diet fed to the piglets.**

Item	Control	Fish Oil
Effective metabolizable Energy, kcal/kg	3844	4115
Crude protein, %	18	17
Crude fat, %	5.1	10
Crude fiber, %	6.2	6.2

Table 2- 2. Fatty acid composition of the fish oil fed to the piglets.

Fatty Acids, %	Composition
Myristic acid	8.04
Palmitic, C16:0	16.85
Palmitoleic, C16:1	11.50
Stearic, C18:0	03.09
Oleic, C18:1	9.74
Linoleic, C18:2	1.89
Alpha linolenic, C18:3	2.20
Stearidonic, C18:4	3.21
Arachidonic, C20:4	2.49
Eicosapentaenoic, C20:5	14.05
Docosapentaenoic, C22:5	2.95
Docosahexaenoic, C22:6	12.26
Other	11.73

Table 2- 3. Production parameters of piglets supplemented with or without fish oil for four weeks.

Item	Control	Fish oil	SEM	Treat
Initial body weight , kg	8.05	8.06	0.19	0.96
Final body weight, kg	11.9	11.3	0.30	0.13
Average daily gain, g	131	115	7.5	0.14

Table 2- 4. Plasma inflammatory mediators of LPS challenged piglets supplemented with or without fish oil for four weeks.

Item	Control	Fish oil	SEM	Treat	<i>P</i> values	
					Hour	Treat × Hour
PGE ₂ , pg/ml	72.8	64.7	3.22	0.08	0.09	0.32
LTB ₄ , ng/ml	0.25	0.24	0.30	0.94	0.05	0.44
TNF- α , pg/ml	817	1318	154	0.03	<0.01	0.13
IL-6, pg/ml	565	395	59.4	0.06	<0.01	0.03

Figures

Figure 2-1. Feed intake on weeks 2 and 4 of piglets supplemented with or without fish oil for four weeks.

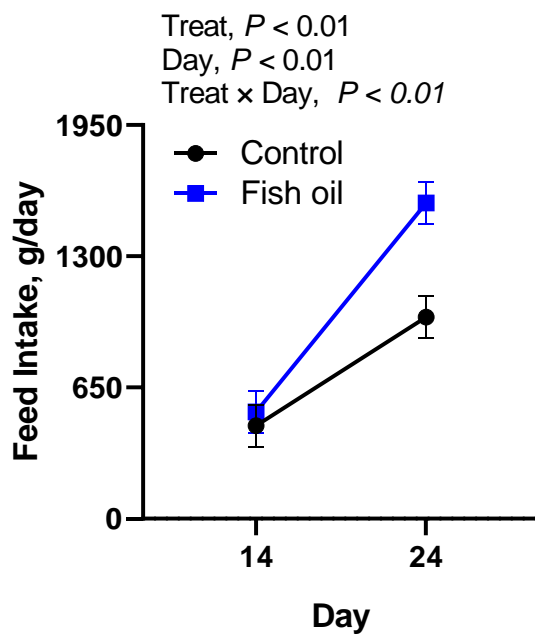


Figure 2-2. Body weight of piglets supplemented with or without fish oil for four weeks.

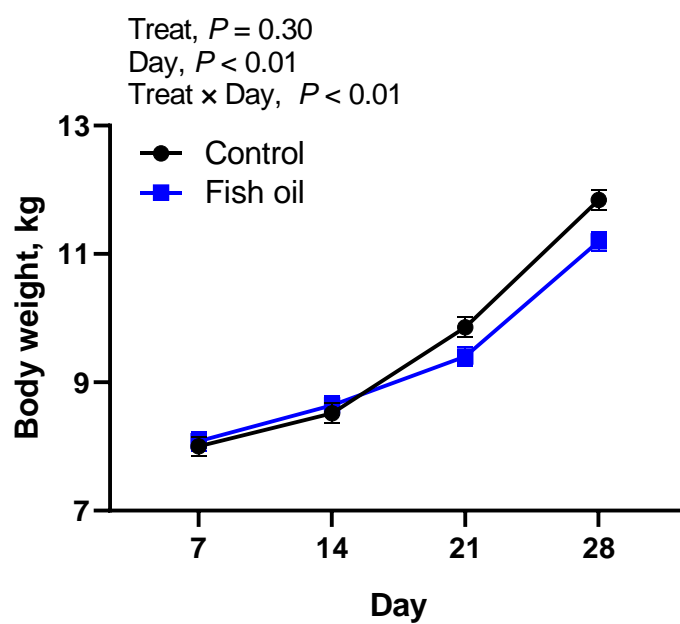


Figure 2-3. Fatty acids digestibility on weeks 2 and 4 of piglets supplemented with or without fish oil for four weeks.

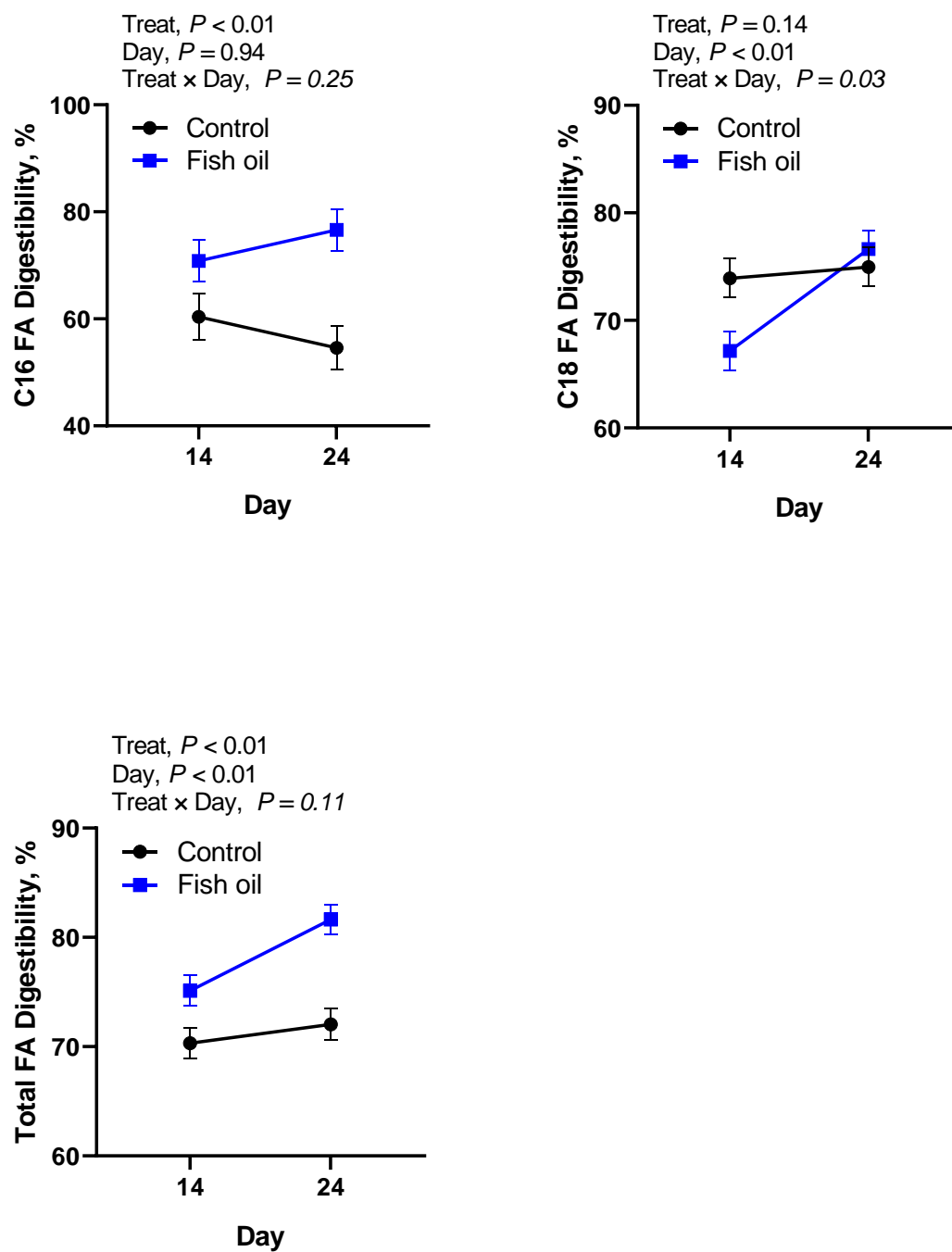


Figure 2-4. Plasma TNF- α concentration of piglets supplemented with or without fish oil for four weeks.

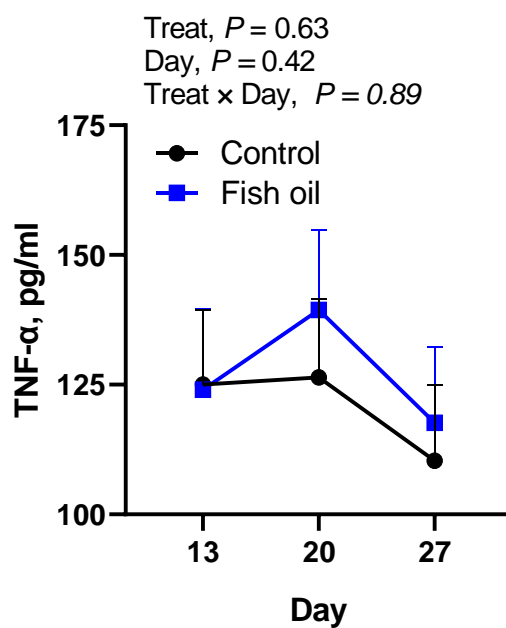
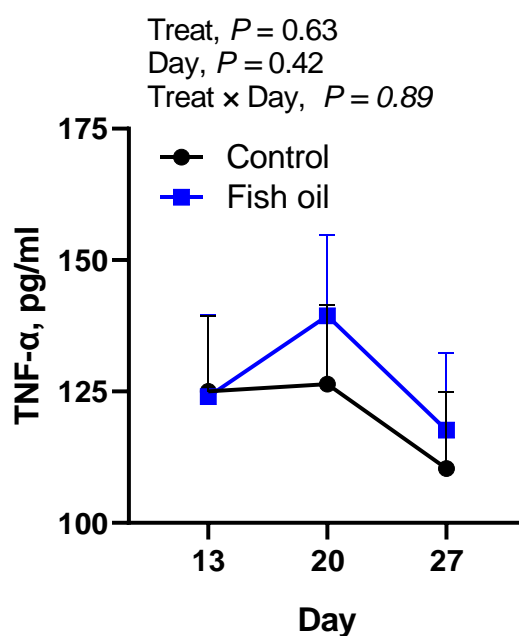


Figure 2-5. Plasma concentration of D-xylose (*in vivo* intestinal permeability) of piglets supplemented with or without fish oil for four weeks.

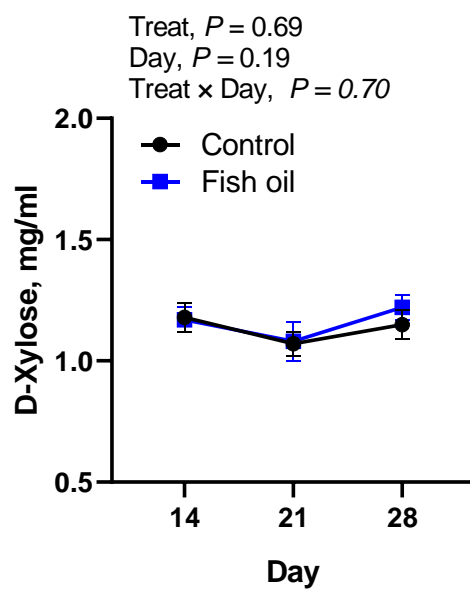


Figure 2-6. Intestinal concentration of volatile fatty acids after the LPS challenge of piglets supplemented with or without fish oil for four weeks

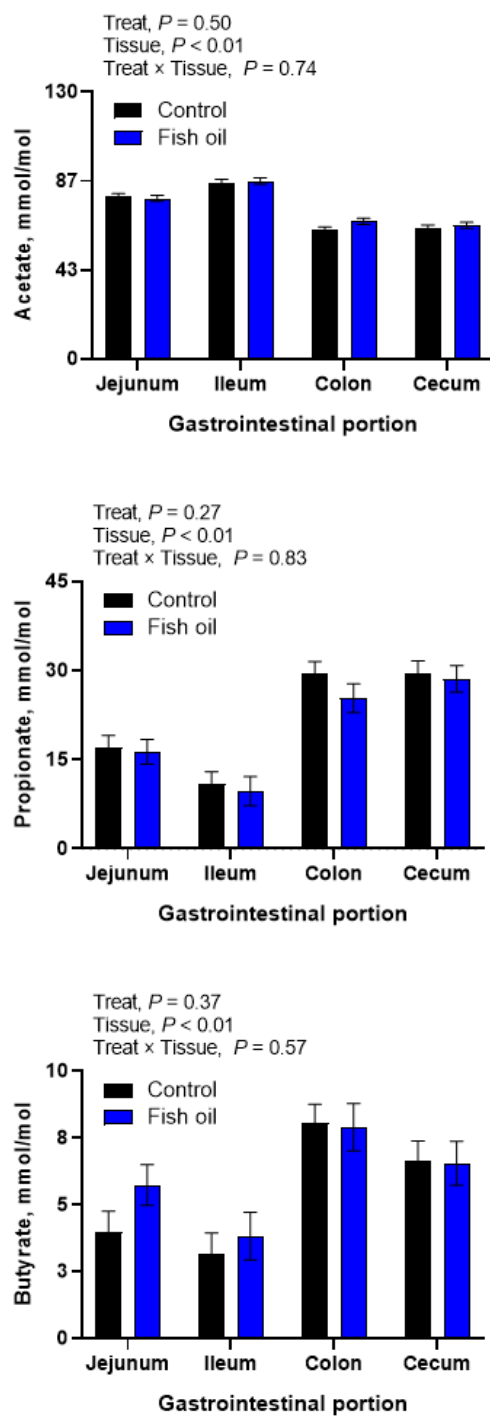


Figure 2-7. Plasma concentration of immune mediators after the LPS challenge of piglets supplemented with or without fish oil for four weeks.

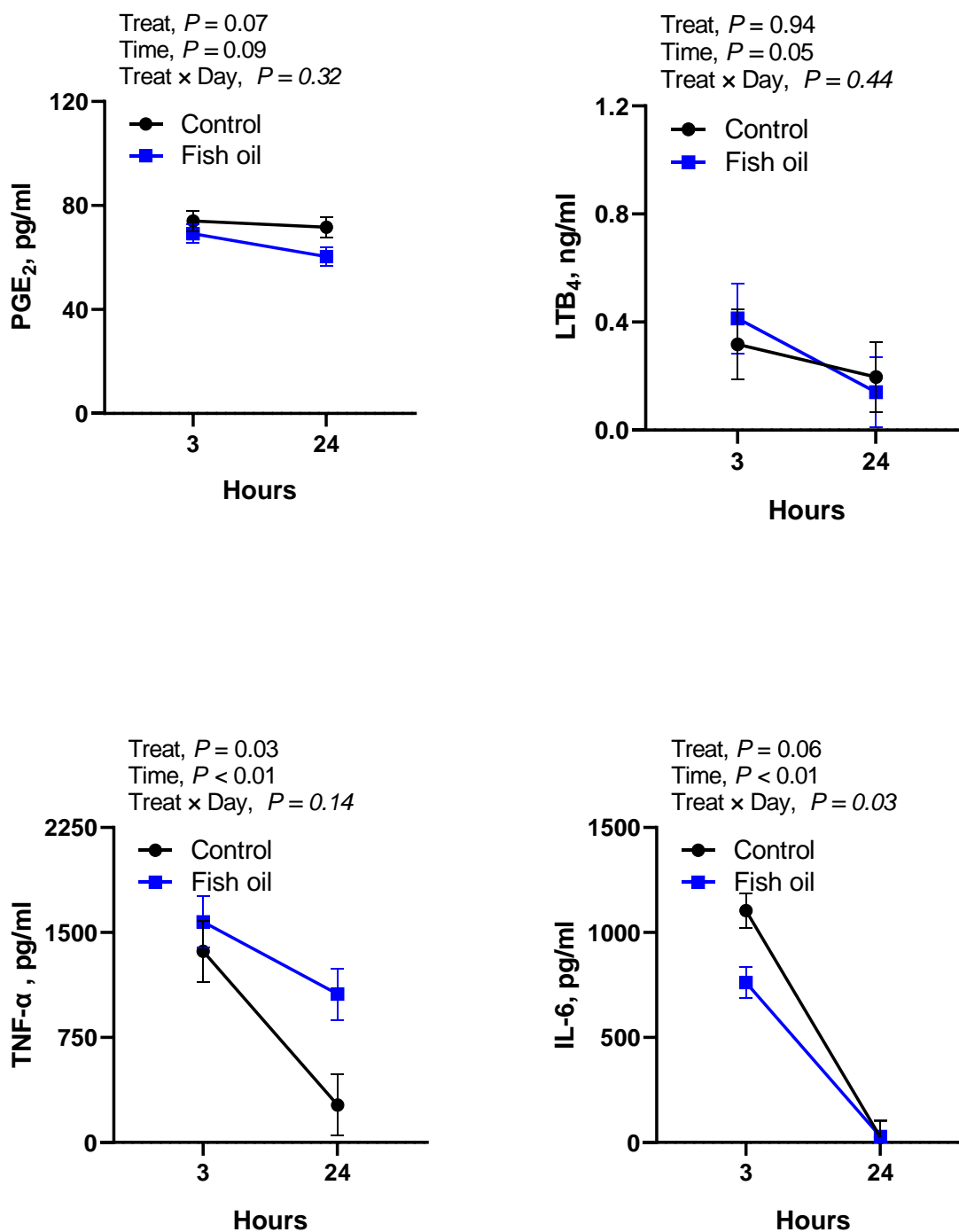
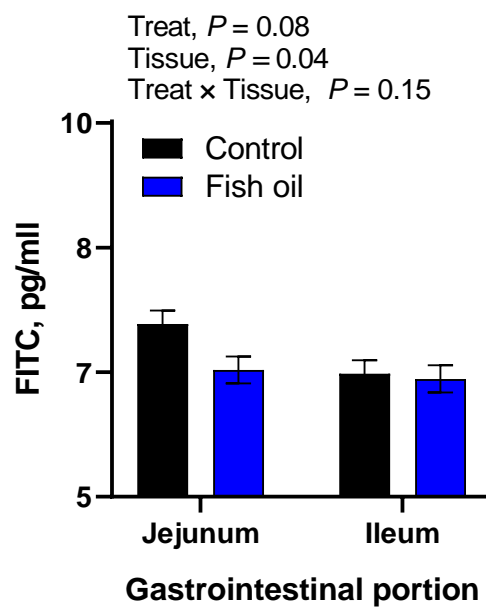


Figure 2-8. The FITC concentration (*ex vivo* intestinal permeability) after the LPS challenge of piglets supplemented with or without fish oil for four weeks.



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